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Title of invention

"Use of soluble protein molecules expressed by the pancreas and kidney glomerulus"
(Haiman ja munuaiskeräsen ilmentämien liukoisten proteiinimolekyylien käyttö)

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Use of soluble protein molecules expressed by the pancreas and kidney glomerulus.

The Technical Field of the Invention

The present invention is related to soluble nephrin-like protein molecules as well as nucleic acid sequences having a substantial similarity with SEQ ID NO:1: and which encode nephrin-like protein molecules which are substantially homologous with human nephrin (SEQ ID NO:2:) but lacking the transmembraneous domain thereof. Said nephrin-like molecules and the nucleic acid sequences encoding them are useful for diagnostic determination, prophylactic and therapeutic treatment of diabetic and other nephropathies, of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases.

The Background of the Invention

The NPHS1-gene and its non-soluble gene-product, named nephrin, found abnormal in patients suffering from congenital nephrotic syndrome of the Finnish type (CNF), an autosomal-recessive disorder characterized by massive proteinuria in utero and nephrosis at birth, has been described by Kestilä, M., et al. (Molecular Cell 1: 575-582, 1998). The NPHS1 gene product is a non-secretable 1241-residue putative transmembrane protein with resemblance to immunoglobulin family of cell adhesion molecules, and exclusively expressed in kidney as reported by Kestilä, M., et al, 1998. The nucleotide derived amino-acid sequence of NPHS1 and its predicted domain structure has also been described in said publication.

The present inventor has identified a new splicing form of the said nephrin encoding mRNA, which is translated into a secretable, soluble nephrin-like molecule. The nephrin-like molecule is expressed and secreted by both kidney and pancreas, indicating that it is not exclusively expressed by kidney. Based on the discovery of this new tissue expression pattern, the present inventor has been able to develop new methods and means for studying, e.g. diabetic and other nephropathies (glomerulopathy), which is one of the most common complications of prolonged diabetes. The treatment of the disorder is also complicated with still undetermined treatment modalities. The ultimate treatment of this and other serious nephropathies with kidney transplantation, is complex, expensive, risky and binds the patients to continuous use of immunomodulatory medication.

Even if it is known that diabetes mellitus is a major cause of end-stage kidney disease, the pathophysiology of especially the glomerular permeability changes in diabetes mellitus remain poorly understood, and little progress has been achieved, in addition to the strict glycemic control, in the targeted treatment of this symptom. Simultaneously with the changing demographics and continuous increase in the incidence of diabetes mellitus, an ever increasing economic burden to health care organizations in all western countries will be evident. Thus, there is a great need of methods and test kits for accurate diagnosis of said disorder as well as methods and means for prophylactic, preventive and therapeutic treatment thereof. In addition, this same applies for other either primary or secondary diseases of the kidney presenting with proteinuria. Moreover, inflammatory, neoplastic, developmental and other acquired diseases of the exocrine and endocrine pancreas have remained poorly characterized often with no known etiopathogenesis.

Thus, the objectives of the present invention is to provide new methods and means for diagnostic determination, prophylactic and therapeutic treatment of diabetic and other nephropathies and of diabetes mellitus and other inflammatory endocrine and neoplastic pancreatic diseases based on the use of the soluble nephrin-like molecules of the present invention, including the splice variant and derivatives of said molecules as well as nucleic acid sequences encoding them.

The present invention provides a solution to the above defined problems by offering new methods and means for diagnosing as well as prophylactic and therapeutic treatment based on the use of soluble nephrin-like molecules and nucleic acid sequences encoding the same.

The Summary of the Invention

The characteristic features of the methods and means are defined in the claims of the present invention.

A Short Description of the Drawings

Fig. 1 Alignment of nucleotide-derived amino acid sequences of rat and human nephrin. The putative transmembrane domain appears in the box, Ig-like modules are marked by shadowing and fibronectin type III-like module is underlined. Cysteine residues identical in human and rat are shown by closed dots and the two additional cysteines of rat are

indicated by arrows (an additional cysteine is found in human locus 974). The cleavage site for predicted N-terminal signal sequence is identical for human and rat as are the putative N-glycosylation sites (not marked).

Fig. 2 Amino acid sequence of nephrin and the respective NPHS1 nucleotides 3121 to 3300 including the transmembrane area (boxed) together with exon boundaries. The missing sequence of the novel nephrin-α splicing variant includes exon 24.

Fig. 3 Immunoblotting of glomerular lysates with antinephrin antibodies shows distinct reactivity with a 200 kD protein band.

The Detailed Description of the Invention

Definitions

In the present invention the terms used have the meaning they generally have in the fields of medicine and diagnostics, especially in bed-side diagnostics as well as immunochemistry. Some terms, however, are used with a somewhat deviating or broader meaning in this context. Accordingly, in order to avoid uncertainty caused by terms with unclear meaning some of the terms used in this specification and in the claims are defined in more detail below.

In the present invention the term "**nucleic acid sequence**" means an isolated nucleic acid sequence encoding nephrin or soluble nephrin-like molecules shared by pancreas and kidney glomerulus and having the "**nucleic acid sequences**" comprise SEQ ID NO:1: or nucleic acid sequences with substantial similarity encoding nephrin-like molecules having an amino acid sequence substantially homologous with SEQ ID NO:2: but lacking the transmembraneous domain GPSGLPLLPVLFALGGLLLLSNASCVGGVL-WQRRLRRL (SEQ ID NO:3:) of nephrin or substantial parts therof. Preferably the nucleic acid should encode a polypeptide having the characteristics described above and at least one contagious amino acid sequence LPTEPPSGISE (SEQ ID NO:4:).

The "**nucleic acid sequences**" of the present invention are not in their natural state but are isolated from their natural environment as expressed mRNAs, which are purified and multiplied *in vitro* in order to provide by technical means new copies, which are capable of encoding said human nephrin or substantially homologous "**soluble nephrin-like molecules**" of the present invention.

The isolated nucleic acid sequences of the present invention also include the human nephrin encoding nucleic acid sequence SEQ ID NO:1:, which is obtainable as a cDNA of mRNA expressed by human pancreas and kidney glomerulus.

The term "**nucleic acid sequence encoding nephrin-like molecules**" means nucleic acid sequences as well as substantially homologous nucleic acid sequences, which comprise at least one contagious nucleic acid sequence, encoding the amino acid sequence, LPTEPPSGISE (SEQ ID NO:4:) overlapping the extra- and intracellular domains of the nephrin molecule, but lacking the nucleic acid sequence encoding the transmembrane domain GPSGLPLLPVLFALGGLLLLSNASCVGGVLWQRRLRRL (SEQ ID NO:3):. As an example of such nucleic acid sequences the contagious sequence CTG CCC ACA-GAG CCA CCT TCA GGC ATC TCA GAG (SEQ ID NO:5:) deduced from human cDNA can be mentioned. This sequence or its complementary sequence or nucleic acid sequences containing said sequence or parts thereof, e.g. fragements truncated at the 3'-terminal or 5'-terminal end as well as such sequences containing point mutations are especially useful as probes for detecting nucleic acid. Specific nucleic acid sequences useful as primers are the sequences for exon 2 were 5'-GAC AAA GCC AGA CAG ACG CAG-3' (SEQ ID NO:6:) and 5'-AGC TTC CGC (SEQ ID NO:7:). It is however clear for those skilled in the art that other nucleic acid sequence capable of encoding nephrin-like molecules and useful for their production can be prepared. The nucleic acid sequences encoding nephrin-like molecules should not be capable of hybridizing under stringent condition ((Sambrook, J., et al., Molecular Cloning: A Laboratory Manual., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989) with sequence encoding the transmembrane domain of the nephrin or parts thereof.

The nucleic acid sequences of the present invention should have a substantial similarity with the SEQ ID NO:1:. "**Substantial similarity**" means that the nucleotide sequences fulfill the prerequisites defined above and have a significant similarity, i.e. a sequence identity of at least 60 %, preferably 70 %, most preferably more than 80 % with the regions of SEQ ID NO:1:, encoding the intra- and extracellular domains, but lacking the region encoding the transmembraneous domain of human nephrin.

The term "**nucleic acid sequences encoding human nephrin or soluble nephrin-like molecules**" include their truncated or complexed forms as well as point mutations of said nucleic acid sequences as long as they are capable of encoding amino acid sequences

having the essential structural features as well as the properties and/or functions of nephrin-like molecules.

The term "**nephrin-like molecules**" means protein molecules or polypeptides, i.e. soluble receptor analogues expressed by pancreas and kidney glomerulus and being substantially homologous to nephrin at amino acid level, but lacking the transmembrane domain of nephrin. Said "**nephrin-like molecules**" are obtainable by isolation from natural sources as transmembrane splicing forms of the nephrin. The splicing forms lack the transmembrane anchoring domain of nephrin and the corresponding mRNA and cDNA lacks said transmembraneous domain encoding nucleic acid sequence or is a nucleic acid sequence from which said region is easily split off. The nephrin-like molecules are also producible by synthetic, semisynthetic, enzymatic and other bio-chemical or chemical methods including recombinant DNA techniques.

The "**nephrin-like molecules**" are substantially homologous with the amino acid sequence SEQ ID NO:2: but lacking the transmembraneous domain, i.e. at least the amino acid sequence between the amino acid Glu human locus 1127 and amino acid Lys human locus 1039, or GPSGLPLLPVLFALGGLLLLSNASCVGGVLWQRRLRRL (SEQ ID NO:3:) of nephrin. The "**nephrin-like molecules**" are preferably characterized by comprising contagious amino acid sequences such as the sequence EDQLPTEPPS-GISEKTEAGSE (SEQ ID NO:8:), LHQPSGEPEDQLPTEPPSGISEKTEAGSEED-RVRNE (SEQ ID NO:9:), LPITTPGLHQPSGEPEDQLPTEPPSGISEKTEAGSEE DRVRNEYEESQW (SEQ ID NO:10:), ADKGTQLPITTPGLHQPSGEPEDQLPTEPPS-GISEKTEAGSEED RVRNEYEESQWTGERDT (SEQ ID NO:11:), ASNALGDSGLAD-KGTQLPITTPGLHQPSGEPEDQLPTEPPSGISEKTEAGSEEDRVRNEYEESQWT-GERDTQSSTVST (SEQ ID NO:12:) or amino acid sequences being truncated at the N-terminal or C-terminal end but still covering the overlapping region LPTEPPSGISE (SEQ ID NO:4:).

The term "**substantially homologous**" at amino acid level means that the nephrin-like protein molecules have a significant similarity or identity of at least 80%, preferably 85 %, most preferably more than 90 % with human nephrin (SEQ ID NO:2:) lacking the transmembraneous domain (SEQ ID NO:3:).

The term "**nephrin-like molecules and derivatives thereof**" comprise polypeptides having the structure, properties and functions characteristic of nephrin-like molecules.

Thus, the term "**nephrin-like molecules and derivatives thereof**" includes nephrin-like molecules, wherein one or more amino acid residues are substituted by another amino acid residue. Also truncated, complexed or chemically substituted, forms of said nephrin-like molecules are included in the term "**nephrin-like molecules and derivatives thereof**". Chemically substituted forms include for example, alkylated, esterified, etherified or amidized forms with a low substitution degree, especially using small molecules, such as methyl or ethyl, as substituents, as long as the substitution does not disturb the properties and functions of the nephrin-like molecules. The truncated, complexed and/or substituted variants of said polypeptides are producible by synthetic or semisynthetic, including enzymatic and recombinant DNA techniques. The only other prerequisite being that the derivatives still are substantially homologous with and have the properties and/or express the functions of the intra- and extracellular domains of nephrin, i.e. the soluble nephrin-like molecules of the present invention. Preferably, all "**nephrin-like molecules and their derivatives**" should be recognizable using binding substances capable of recognizing the natural human nephrin or nephrin-like molecules. The term "**nephrin-like molecules**" otherwise covers all possible splice variants of nephrin expressed by pancreas and kidney. The term "molecules or proteins shared by pancreas and kidney" means that the protein is expressed by cells of both pancreatic and kidney tissue, it is they have the same tissue expression pattern.

As a conclusion "**nephrin-like molecules**" in its broadest aspect in the present invention, covers not only nephrin-like molecules derived from nature, including their isoforms of different origin, but also synthetically, semisynthetically, enzymatically produced nephrin-like molecules including molecules produced by recombinant DNA techniques. Said nephrin-like molecules can be used either as separate entities or in any combinations thereof.

The term "**isoform**" refers to the different forms of the same protein, which originate from different sources, e.g. different species. In the present invention the term, thus, includes fragments, complexes and their derivatives. For example, nephrin-like molecules can be generated by the cleavage of the proprotein. Different reactions, including different enzymatic and non-enzymatic reactions, proteolytic and non-proteolytic, are capable of creating a truncated, derivatized, complexed forms of the molecules.

The amount of nephrin-like molecules are preferably determined with "**binding sub-**

stances". The term "binding substance" means any substances capable of specifically recognizing and binding the soluble nephrin-like molecule or derivatives therof or at least one portion in the intra- and/or extracellular domain of nephrin or both. Preferably, the binding substance should recognize simultaneously both domains of nephrin, but not the transmembrane anchoring domain. Such substances are, for example, receptors or binding proteins or peptides, capable of specifically binding said nephrin-like molecules, but above all they mean antibodies capable of specifically recognizing one or more nephrin-like molecules alone or in any combination. The antibodies include both polyclonal and/or monoclonal antibodies as well as fragments or derivatives thereof. Preferably, binding substances recognizing and binding sequence specific epitopes or active sites of the nephrin-like molecules should be chosen.

Said binding substances can be produced using the intra- and extracellular domains of nephrin or any nephrin-like molecules, their isomers as well as their fragments, derivatives and complexes with the prerequisite that they are capable of acting as "**antigens**", in other words, antigens include any compositions or materials capable of eliciting an antibody response specific to said nephrin-like molecules. Said binding substances, preferably antibodies are producible by conventional techniques for producing polyclonal antibodies as well as monoclonal antibodies. The methods for preparing monoclonal antibodies include hybridoma techniques. Fragments of antibodies or other binding proteins like specific binding peptides can be developed by phage display techniques and produced by recombinant DNA techniques. All methods are well known by those skilled in the art and described in laboratory handbooks.

The term "**diagnosing**" means judging, predicting, assessing or evaluating from the recorded results if a person is susceptible of or suffers from diabetic and other nephropathies and of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases. The diagnoses also enable evaluation of the severity of the condition, therapy required as well as the efficacy of treatment modalities or medical treatment. Especially, early identification of diabetes in order to start prophylactic dietary or other treatments before the onset of the actual disease is a desirable feature, enabled by the present invention.

The results are recordable with means for performing immunoassays using nephrin-like molecules and/or their binding substances as well as parts thereof or means for performing amplification and hybridization methods using sequence specific probes or primers, which can be selected from the parts of SEQ ID NO:1: encoding the intra- and/or extracellular domains of human nephrin.

The term "screening a population for the presence or absence of autoantibodies" means that based on the fact that people susceptible of diabetes mellitus and complications related to said disease produce antibodies against nephrin-like molecules, which can be determined using nephrin-like molecules, it is easy to screen a large population for the susceptibility of the diseases mentioned above from blood or serum samples obtained from inflowing serum samples for routine diabetes diagnosis. If such autoantibodies can be detected in the serum of a person, it is a clear indication that the person in question is a potential diabetes patient and might be susceptible also to diabetic kidney diseases and should be treated appropriately.

The term "**immunoassay**" refers to a method or procedure capable of detecting and/or measuring at least one substance, either nephrin-like molecules or autoantibodies against said molecules using per se known means for performing an immunoassay, which means including a substance capable of specifically recognizing the substance to be determined, i.e. either at least one binding substance or a nephrin-like molecule or fragments thereof, for the desired application, respectively.

Well known examples of immunoassays are radioimmunoassays (RIA), radioimmunometric assays (IRMA), fluoroimmunometric assays (IFMA) enzyme immunoassays (EIA), enzyme-linked immunosorbent assays (ELISA), fluoroimmmunoassays (FIA), luminescence immunoassays, immunoagglutination assays, turbidimetric immunoassays, nephelometric immunoassays, etc. All methods are well known by those skilled in the art and described in laboratory handbooks.

The preparation and development of methods and means for measuring different antigens and antibodies, which can be applied also to the determination of nephrin-like molecules and their autoantibodies have been described for example in the following patent publications applicable as convenient bed-side kits US 5,591,645, US 5,712,170, US 5,602,040, US 5,622,871, US 5,656,503, EP 149 168, US 4,552,839, US 4,361,537, US 4,373,932, WO 86/04683, EP 154 749, EP 7654, WO 86/03839, EP 191 640, EP 212 599, US 4,552,839, EP 158 746, EP 225 054 and which are herewith incorporated by reference. Even if said patent is restricted to the development of test kits for diagnosing other diseases by aid of binding substance recognizing the respective active molecule, the man skilled in the art can use the information for developing corresponding test kits for measuring nephrin-like molecules of the present invention.

Any immunochemical test methods can in principle be used for diagnosing diabetic and other nephropathies, of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases as well as for longitudinal or latitudinal screening of the progress of disease and effect of medical treatment. However, visual agglutination, flow-through and immunochromatographic methods are best suited for rapid bed-side assays or tests.

"**Bed-side assays**" refer to tests or procedures which can be carried out without any laboratory facilities and without the need of qualified laboratory personnel. Bed-side tests can be made by the physician, while the patients are visiting the doctors or when the doctors make their daily bed-side visits to the patients. "**Bed-side assays**" are preferably performed on "**solid carriers**" like test strips. Such bed-side assays are based on immunochemical and hybridization techniques and several applications have been developed and described in the literature. The only prerequisite for the skilled person to develop new methods and tools (test kits) is to provide suitable antigens, antibodies for the immunoassays and suitable probes and primers for the hybridization and PCR-techniques. In the present invention such antigens, antibodies, probes and primers are disclosed.

The term "**prophylactic treatment**" includes specific dietary measure and/or precautions, e.g. glycemic control including possible medication, before the onset of diabetes. After the on-set of diabetes, "**prophylactic treatment**" requires therapeutic treatment of diabetes as a precaution in order to avoid kidney complications.

The term "therapeutic treatment" includes methods for treating persons with administration of the soluble protein product, gene therapy or preventing the genes causing the disease from expressing the gene products causing the diseases, including diabetic and other nephropathies and of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases.

The General Description of the Invention

Renal involvement of poor glycemic control is a major complication of diabetes mellitus (Kanwar, Y.S., et al., Semin Nephrol 11:390-413,1991; Rennke, H.G.: Kidney Int 45:S58-63, 1994; Fioretto, P., et al., N Engl J Med 339:69-75, 1998). The diabetic nephropathy is characterized by a gradual and worsening loss of the glomerular filtration barrier function, increase of glomerular basement membrane thickness and accumulation of amorphous material within the glomerular mesangium, composing the typical Kimmel-

stiel-Wilson pathology of glomeruli (Kanwar, Y.S., et al., Semin Nephrol 11:390-413,1991; Rennke, H.G., Kidney Int 45:S58-63, 1994.). Simultaneously with the loss of glomerular functions, deterioration of renal tubular functions has been found.

However, little is presently known of common epitopes shared with the kidney glomerulus and the islets of Langerhans of pancreas. The solid demonstration of success in the combined pancreas-renal transplantation offers still another exciting new alternative explanation: that shared functional epitopes are involved. Thus far only anecdotal molecules including Pod-1 (Quaggin SE, et al., Mech Devel 71:37-48, 1998), endothelin-B receptor (Yamamoto T. & Uemura H:, J Cardiovasc Pharmacol 31:S207-211, 1998) and some sulphated glycolipids (Buschard K, et al., APMIS 101:963-70, 1993) are reportedly shared between kidney and pancreas but are also shared in a variety of other tissues and thus not particularly promising functional pathogenic epitopes.

Results of the present inventor indicate that nephrin is distinctly down-regulated in the human proteinuric disease of congenital nephrotic syndrome and is dramatically down-regulated in the experimental puromycin model mimicking the human "minimal change" pure proteinuria (Luimula, P., et al., submitted). At the same time antipeptide antibodies to the respective protein product indicate that the protein is expressed in glomerular podocytes, the cells crucial for glomerular permeability (Ahola, H. et al., submitted). Surprisingly, the only other tissue site expressing both the gene and its protein product in a survey of 60 human cells and tissues was the islets of Langerhans. The initial results of the inventor indicated that both the gene and the respective protein is found and regulated also in insulin-producing cell lines (Ahola, H., et al., manuscript in preparation) including the rat insulinoma cell RIN, AR42J and INS-cells. Furthermore, it was shown that antibodies to this gene product are characteristically found in both human diseases with proteinuria (Wang, et al., in preparation) and distinctly in prediabetics with high circulating antibody titres to glutamic acid decarboxylase (GAD65) and pancreatic Langerhans islet cell (ICA) antibodies.

Accordingly, the recently identified new tissue expression pattern and posttranscriptional splicing of the gene, which previously was believed to be uniquely expressed by kidney indicates that the gene product is expressed in a unique way only in the endocrine and exocrine cells of pancreas and kidney glomerulus. Antibodies raised against the protein product encoded by the gene undisputably shows that it exists primarily in pancreas and kidney. Approximately 60 % of the patients suffering from prediabetes (so called islet

cell -and glutamic acid dehydrogenase-(GAD)-antibody positiveness) as well as patients suffering from certain kidney diseases produce antibodies against this molecule.

In addition, the present inventor has identified a form of splicing of the nephrin coding mRNA which yields a secreted soluble receptor analogue of this protein in kidney and pancreas. Because the splicing variant is among the first proteins shared by the kidney and pancreas, it is especially promising for producing future diagnostic methods as well as for drug development and therapeutic treatments, such as the use of the soluble receptor analog to modify disease and for gene therapy. Based on said findings it is also possible to develop similar nephrin-like molecules and derivatives thereof which have the same properties and functions.

The characterization of the structure of the transmembrane splice form of the gene and the clarification of its molecular function in kidney and pancreas has shown that the gene is critically involved in the regulation of kidney glomerular permeability barrier. NPHS1 and its respective gene product are present within the pancreatic acinar cells and in the islet cells of Langerhans. Extraordinarily, the kidney glomerulus and pancreas are the only cell types among the 60 different human tissue sites tested for positivity in a Northern blot for the specific mRNA expression. Furthermore, the present inventor has shown that this gene is distinctly expressed within various insulin producing cell lines (AR42J; RINm5F; INS-1) as well as in the isolated porcine and human pancreatic islands. The insulin secretagogue stimulation of the cell lines up-regulates nephrin expression. Furthermore, nephrin protein serves as an autoantigen in a group of islet-cell antibody (ICA) and glutamic acid decarboxylase (GAD65) positive prediabetics and is similarly down-regulated in various proteinuric kidney diseases showing that a completely new functional molecule shared with the glomerular filtration barrier and functionally crucial of the pancreas is involved.

Based on the findings disclosed above it can be concluded that the nephrin-like molecules of the present invention, the nucleic acid sequences encoding them as well as the binding substances specifically recognizing them are highly promising molecules to understand the pathophysiology of e.g. diabetic nephropathy. The present invention provides isolated and essentially purified novel nucleic acid sequence defined above. The nucleic acid sequence covers at least part of the intracellular and the extracellular portion of the NPHS1-gene, but lacks the portion of the NPHS1-gene expressing the transmembrane anchoring portion of nephrin.

The nephrin-like molecules are soluble polypeptides or proteins and as such especially useful for detecting and/or determining the presence or absence of autoantibodies when screening people for susceptibility of diabetic and other nephropathies, of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases.

The binding substances, which are capable of specifically recognizing and binding the soluble nephrin-like molecules, are preferably such that are capable of specifically recognizing either at least one epitope in the intra- and/or the extracellular domain of nephrin or preferably an epitope covering both domains but not the transmembraneous domain.

The molecules described above, including nephrin-like molecules, nucleic acid sequences encoding them and binding substances specifically recognizing them are useful for diagnostic determination, prophylactic and therapeutic treatment of diabetic and other nephropathies and of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases as well as for screening a multitude of samples in order to evaluate if a person is susceptible of diabetic or other nephropathies, of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases.

The present invention is related to a diagnostic kit for determining whether a subject is a nephropathy disorder carrier or a patient having a nephropathy associated disorder, which kit includes at least one nephrin-like molecule capable of recognizing and specifically binding to autoantibodies from serum samples obtained from patients susceptible of said diseases as well as packing means and instructions for use.

Alternatively, the invention is related to a diagnostic kit for determining whether a subject is a nephropathy disorder carrier or a patient having a nephropathy associated disorder, which kit includes at least one binding substance capable of recognizing and specifically binding to nephrin-like molecules from samples obtained from patients susceptible of said diseases as well as packing means and instructions for use.

The nephrin-like molecules and the corresponding autoantibodies can be assessed by various known methods. The the levels of nephrin-like molecules in blood samples obtained from healthy and sick persons can be estimated using their specific antibodies. Autoantibodies and/or nephrin-like molecules can be identified by Western blotting. After SDS-PAGE of the preparations, the bands can be transferred onto nitrocellulose and characterized by staining with polyclonal antibodies specific to said nephrin-like

molecules. With methods like this, specific results indicating correlation of severity of disease and medication needed can be obtained, but said methods are far too laborious and time consuming to be used routinely. Moreover, it is impossible to develop a rapid bed-side test based on any kind of electrophoresis. The diagnostic determination of the present invention should be aimed at evaluating the disease from a blood sample, in order to be able to give appropriate treatment regimen at the right time, as well as to monitor the effect of therapy and treatment modalities as well as the prognosis of the disease. A higher level of nephrin-like molecules means a more severe disease and the need of a more guarded prognosis and medical treatment as evidenced by the present invention. Specific therapy regimens are related to disease severity and actual activity.

The antibodies raised against nephrin-like molecules and being capable of specifically recognizing them, were used by the present inventor in immunoblot analysis of blood samples obtained from sick and healthy persons. Various representatives of poly- and monoclonal antibodies recognizing nephrin-like molecules listed below can be produced by *per se* known methods. Monoclonal antibodies of the present invention can be and have also been developed according to the original technique of Köhler and Milstein (Nature 256, 495, 1975).

Based on the results obtained and the antibodies available the present inventor developed new methods and test kits for an effective, rapid, and reliable assessment of the status and tissue destruction status as well as to identify the phases of disease activity in pancreas and kidneys of human beings. The methods and test kits of the present invention are based on the fact that there is a relation especially between the presence of nephrin-like molecules and autoantibodies against said nephrin-like molecules and susceptibility and the severity of disease activity. Also indicated is the fact that certain nephrin-like molecules alone or in any combination are more specific than others in assessing the disease and that there is some differences in specificity and selectivity, too. Hence, it is advantageous to develop kits by which a multitude of nephrin-like molecules alone or in any combination could be determined simultaneously, either on the same test strip or on separate test strips. In preferred embodiments the nephrin-like molecules, shown to be most suitable or effective for a specific diagnostic purpose, were selected for the test kit, either alone or in any combination.

Different numeric results obtained in the experiments reflect the use of different sets of binding substances or antibodies as well as other variation in the test conditions. Howev-

er, it is important to notice that even if the level of nephrin-like molecules differ in different persons, the ratio between diseased and healthy persons remains approximately the same and a qualitative, semiquantitative or even quantitative test can be developed for bed-side assessment of the severity of the diseases. The results can be recorded visually or by a recording instrument either directly or indirectly by adding a substrate capable of making the binding reaction recordable.

These findings indicate that an immunochromatographic test using monoclonal antibodies which capture and recognize nephrin-like molecules alone or in any combination, is fully sufficient for diagnosing disease activity. The invention disclosed in the present patent specification provides a highly effective diagnostic tool for an accurate evaluation of the kind of treatment and regimen of therapy needed. The methods and test kits of the present invention also provide alternative bed-side diagnostic tools for evaluating the severity of the disease. At the same time the methods and test kits provide effective tools for follow up studies of the efficacy of the therapy or treatment as well as the dose-treatment response obtained.

Based on these founding methods and test kits were developed for diagnosing the level, and/or severity of the disease processes, evaluating the efficacy of drug treatments, other treatment modalities, other medications and/or predicting the risk for progress of said diseases, wherein the detection is performed as a rapid and reliable immunological bed-side assay using blood samples from which one or more nephrin-like molecules alone and/or in combination can be measured.

The nucleic acid sequences of the present invention and defined above, especially SEQ ID NO:1: can be used to produce suitable primers and probes to be used in *per se* known hybridization techniques and PCR-techniques, some of which are also commercially available, are described in literature, patent publications, laboratory handbooks and can be adapted to the purposes of the present invention. The PCR-techniques for amplifying, detecting and/or cloning nucleic acid sequences were first described and patented by Mullis, K.B. et al. for example in the European Patents EP 200 362 and EP 201 184. Suitable primers and probes for amplification and/or nucleic hybridization techniques are parts of the nucleic acid sequence SEQ ID NO:1:, excluding any parts hybridizing with the nucleic acid sequences encoding the transmembraneous domain of nephrin. The nucleic acid sequence used as primers and probes should comprise at least 10 nucleotides complementary to 10, preferably 15, most preferably 20 consecutive nucleotides from the above-defined sequence.

The nucleic acid sequence described above can be copied and inserted into suitable recombinant expression vector, capable of expressing the gene product when transferred to a suitable host in order to produce recombinant nephrin-like molecules useful in diagnostic kits. The nucleic acid sequences of the present invention are useful in methods for screening people to determine whether said subject is a carrier of the gene causing the disorder, i.e. a nephropathy or associated disorder gene carrier. The method comprises detecting the presence or absence of the nucleic acid sequences of the present invention. Thus, the invention is related to a diagnostic test kit for amplifying a portion of a nucleic acid sequence, a pair of nucleic acid primers complementary to a portion of nucleic acid sequence defined above as well as packaging means and instructions for use.

Also targeting recombinant expression vector can be produced by inserting said nucleic acid sequences and functionally inert modification thereof into the vectors. Such targeting vectors can be used in gene therapeutic applications. The targeting recombinant expression vectors are either modified so that they do not allow expression of nephrin-like molecules when integrated with the gene responsible for diabetic and other nephropathies, of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases.

The inventions is related to a diagnostic kit for determining whether a subject is a nephropathy disorder gene carrier or a patient having a neuropathy associated disorder, which kit includes as probes nucleic acid sequences capable of hybridizing under stringent conditions to nucleic acid sequence describe above as well as packing means and instructions for use. The results are directly applicable to patient diagnostics, and the full diagnostic, prognostic and pharmacologic lead-compound identification dimensions can thus far only be speculated.

The invention is related to a method for treating a patient afflicted with a nephropathy disorder which method comprises targeted administering a nucleic acid sequence and to a method for treating or preventing a diabetic and other nephropathies. The method comprises for example administering to a patient in need thereof a gene having the sequence according to so as to prevent expression of the protein.

Despite the increased availability of genetically modified mouse strains, the experimental models in the rat have provided the most widely employed and versatile models to study diabetic and other nephropathies and diabetes mellitus and other inflammatory and neoplastic pancreatic diseases including their pathophysiology and functional genetics. The

present inventor has cloned and characterized the rat nephrin cDNA. The rat cDNA has an open reading frame of 3705 bp, and shows 82 % sequence identity to the human nephrin cDNA and shows characteristic rat specific splicing variants. The translated nucleotide sequence has 89 % sequence identity at the amino acid level. The signal sequence, glycosylation and cystein localization patterns are nearly identical with those of human nephrin. Like the human, the rat nephrin transcript is expressed in a tissue restricted pattern. Thus, preparation of transgenic animals are enabled by the present invention.

Experimental models of diabetic and other nephropathies and diabetes mellitus and other inflammatory and neoplastic pancreatic diseases are important tools to investigate the functional significance of novel molecules by providing a means to effectively modulate the basic functions. Thus, even if transgenic and knockout mouse models are extremely useful in the targeted analysis of molecules, the well established experimental models in the rat remain best characterized and most widely used for studying pathophysiology of diabetic and other nephropathies and diabetes mellitus and other inflammatory, immunologic and neoplastic pancreatic diseases. Thorough understanding of this gene encoding nephrin and nephrin-like protein molecules as well as its regulation and functions are imperative also for developing targeted therapeutic actions.

The present invention is further described in the following examples, which should not be interpreted as restricting the scope of the protection. The feasibility and applicability of the methods and test kits of the present invention are evident to a man skilled in the art to develop other equally well functioning applications for diagnostic and therapeutic use.

Example 1
Antipeptide antibodies to nephrin
Design of synthetic peptides.

Sequence specific intracellular (aa 1101-1126) and extracellular (aa 1039-1056) oligopeptides were selected over the human nephrin sequence (Gene bank accession number AF035835) using the PredictProtein program via Internet at European Molecular Biology Laboratory, (Heidelberg, Germany). These peptides showed no homology to other known protein sequences and were synthesized and purified at a local peptide synthesis unit (Haartman Institute, University of Helsinki).

Example 2
Antipeptide antibodies

For immunizations the peptides were coupled to a multiple antigenic peptide-polylysine matrix) and injected in two rabbits each. First immunization was with 500 μg of peptide in Freunds complete adjuvant (Difco laboratories, Detroit, Mi), and two booster immunizations with 300 μg each with Freunds incomplete adjuvant four weeks after the previous immunization. Peptide specific fractions were immunoaffinity purified on CNBr-Sepharose (Pharmacia, Uppsala, Sweden) coupled to the corresponding linear peptides. The specificity of the antisera was tested by immunofluorescence (IF) on kidney sections with and without free peptide competition (Fig. 1a), by immunoblotting of glomerular extracts and precipitation of a full length nephrin in an *in vivo* transcription and translation assay using the specific antibodies (see below).

Example 3
Electrophoresis and Western blotting.

For SDS-PAGE the detergent extracts of isolated human glomeruli were suspended in the Laemmli sample buffer, boiled for 5 min, and run under reducing conditions using 8 % gels and a Protean Mini-gel electrophoresis system (Bio-Rad Laboratories, Richmond, CA). The separated proteins were transferred to nitrocellulose sheets for Western blotting using a Novablot semidry blotting apparatus (Pharmacia). After blocking with 3 % BSA the nitrocellulose strips were incubated with the respective antibodies, washed thoroughly and further incubated with anti-rabbit IgG coupled to horseradish peroxidase. After washing the bound antibodies were detected using the ECL^{TM} Western blotting kit (Amersham Pharmacia Biotech).

Example 4
Immunofluorescence and immunoelectron microscopy.

Samples from normal human cortical tissue were prepared for immunofluorescence. Briefly, frozen cortical tissues were cut at 4 μm, fixed in acetone at -20 °C for 10 min and reacted with the antipeptide antibodies (1.1 mg/ml; used at 1:100 dilution in PBS) for 1 hour. FITC-antirabbit IgG (Boehringer, Mannheim, Germany) was used as second antibodies. As control, the primary antibodies were either omitted or replaced by irrelevant antipeptide antibodies. An additional control included preincubation of the antibody with a dilution series of the oligopeptide used as the original immunogen. Postem-

bedding electron microscopy was done using CNF and normal kidney cortical kidney samples fixed in freshly prepared 4% formaldehyde in PBS and embedded in Lowicryl K4M (Chemische Werke LOW1, Waldkraiburg, Germany) and further incubated with the rabbit antinephrin antibodies (50 μg/ml) and the respective 10 nm gold conjugate (1:50).

Example 5
***In vitro* transcription/translation.**

In vitro transcription/translation of full length nephrin sequence under T3 promoter of pBK-CMV (Promega, Madison, WI) was performed according to the manufacturer's instructions with TNT T3 Coupled Reticulocyte Lysate System (Promega, Madison, WI) - a single tube modification of rabbit reticulosyte lysate translations (22). During translation nephrin was labeled with [^{35}S]methionine (NEN Life Science Inc., Boston, MA). Intracellular antibodies (see above) and [^{35}S]labeled nephrin were incubated without or with increasing amounts (0.1, 1 and 10 μg) of intracellular peptide overnight at 4 °C. Immunocomplexes were collected with protein A - Sepharose (Zymed Laboratories Inc. San Francisco, CA) by incubating immunocomplexes and protein A-Sepharose 45 minutes at 4 °C. After incubation the immunocomplexes were washed eight times with washing buffer (150 mM NaCl, 20 mM TRIS (pH 7.6), 0.15% Tween 20, 0.1% BSA, 0.02% sodium azide), and the radioactivity of precipitates was measured in scillation liquid OptiPhase SuperMix (EG&G Wallac, Finland) with 1450 MicroBeta Trilux Liquid Scillation&Luminescence Counter (EG&G Wallac, Finland).

Example 6
Immunoprecipitation.

For immunoprecipitation the glomerular lysate (1 mg/ml) in RIPA buffer was incubated with rabbit or mouse antibodies against occludin (Zymed Laboratories Inc., San Francisco, CA) or ZO-1 (Zymed) using 10 μg of IgG/200 μl of glomerular lysate at 4 °C overnight. Immune complexes were collected with protein-A-Sepharose (Pharmacia LKB Biotechnology), washed and processed for immunoblotting with antinephrin antibodies as described above.

Example 7
Mutation analysis.

Analysis of the Fin_{major} (in exon 2) and Fin_{minor} (exon 26) mutations in the only patient sample out of 28 studied showing immunoreactivity with the antinephrin antibodies was done. Briefly, after DNA isolation, the respective exon areas were amplified by PCR using AmpliTaq DNA polymerase (Perkin Elmer) and the following conditions: initial denaturation at 94 °C for 12 min, 30 PCR cycles (95 °C 1 min, 60 °C 1 min and 72 °C 1 min) followed by final elongation at 72 °C for 8 min. The primer sequences for exon 2 were 5'-GAC AAA GCC AGA CAG ACG CAG-3' (SEQ ID NO:6:) and 5' AGC TTC CGC (SEQ ID NO:7:).

Example 8
Characterization of the gene and the gene product

Conventional immunohistochemical methods, in-situ hybridization and Northern blotting, and well as semiquantitative RT-PCR and electron microscopy can be performed with methods already available and previously used. Our glomerulus-pancreas-gene and its product can be used as a novel pathogenic epitope of kidney diabetic complications, being an imperative factor for a thorough understanding of this gene, its regulation and functions are imperative also for developing targeted therapeutic actions.

Example 9
Analysis of autoantibodies; *Autoantibodies to gene product:*
Determination and characterization of the autoantibodies in diabetes and proteinuria.

The already existing first results of autoantibody production in diabetic patients will be expanded systematically and includes an analysis of antibodies in the sera available from the DiMe Research Consortium or some other collaboration axis.

Another source for expanding the patient sera derives from the accumulating samples for the analysis of islet cell (ICA) and glutamic acid dehydrogenase (GAD65) antibodies at HUCH-Diagnostica (500-800 samples/year). Particularly, the data so far show that 45-60 % of the elevated ICA and GAD positive sera are positive with our kidney- pancreas-epitope, and a careful analysis of patient data will be needed to characterize this patient group. Also, the outcome of diabetic patients treated with kidney transplantation and analyzing their antibody levels will be of utmost importance.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Holthofer, Harry
(B) STREET: Puolaharju 26 B
(C) CITY: Helsinki
(E) COUNTRY: Finland
(F) POSTAL CODE (ZIP): FIN-00930

(ii) TITLE OF INVENTION: THE USE OF SOLUBLE PROTEIN MOLECULES EXPRESSED BY THE PANCREAS AND KIDNEY GLOMERULUS

(iii) NUMBER OF SEQUENCES: 12

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3705 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(F) TISSUE TYPE: Pancreatic islets, kidney glomerulus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATGTCCAGTT TGACTCCCCT GCTGCTCATG GGAATGCTGA CCTCAGGCCT GGCCGAGTCG 60

CCAGTCCCCA CCTCAGCACC TCGAGGCTTC TGGGCTCTGT CTGAAAACCT GACTGCGGTG 120

GAAGGGACAA CAGTTAAGCT ATGGTGCGGT GTCAGGGCCC CTGGCAGTGT GGTGCAGTGG 180

GCTAAGGATG GGCTGCTTCT GGGTCCAAAC CCGAAGATGC CAGGCTTCCC GAGGTACAGC 240

CTGGAAGGAG ATCGTGCTAA AGGCGAGTTC CACCTGCTTA TTGAAGCCTG TGACCTCAGT 300

GATGACGCAG AGTATGAATG CCAAGTCGGC CGCTCAGAGT TGGGTCCCGA GCTTGTGTCT 360

CCTAAAGTAA TCCTCTCCAT TCTAGTTTCC CCCAAGGTGC TTCTGTTGAC CCCCGAGGCA 420

GGAAGCACAG TGACCTGGGT AGCTGGGCAG GAGTATGTGG TCACCTGTGT GTCTGGGGAT 480

GCAAAACCAG CACCTGACAT CACCTTCATC CAGAGTGGAC GAACTATATT GGACGTCTCC 540

TCCAATGTGA ATGAGGGATC AGAGGAGAAA CTCTGCATCA CAGAGGCCGA AGCCAGGGTG 600

ATACCCCAGA GCTCGGATAA CGGGCAGTTA CTGGTCTGTG AGGGTTCCAA CCCAGCTTTG 660

GACACTCCCA TAAAGGCTTC ATTCACCATG AATATTCTGT TTCCCCCAGG ACCTCCTGTC 720

ATTGATTGGC CAGGCCTGAA TGAAGGGCAT GTGAGGGCAG GGGAGAACCT GGAGCTGCCC 780

TGCACAGCCA GAGGTGGCAA TCCACCTGCT ACCCTGCAGT GGCTGAAGAA CGGTAAACCA 840

GTGTCCACAG CCTGGGGCAC CGAGCATGCC CAGGCAGTGG CCCACAGTGT GCTGGTGATG 900

ACTGTACGAC CTGAAGACCA TGGAGCTCGG CTCAGCTGTC AGTCCTACAA CAGCGTGTCT 960

GCAGGGACCC AGGAGAGAAG CATCACACTA CAGGTCACCT TTCCCCCAAG CGCCATTACC 1020

ATCCTGGGAT CTGTATCACA ATCGGAGAAC AAGAACGTGA CCCTTTGCTG CCTGACCAAG 1080

TCCAGTCGCC CACGGGTCCT GCTGCGATGG TGGTTGGGTG GACGGCAGCT GCTGCCCACA 1140

GATGAGACAG TCATGGATGG CCTGCATGGT GGCCACATCT CCATGTCCAA TCTCACATTC 1200

TTGGTGCGGA GAGAAGACAA TGGCCTGCCC CTCACGTGTG AAGCCTTCAG TGACGCCTTC 1260

AGCAAGGAGA CCTTCAAGAA GTCACTCACC TTGAATGTGA AATACCCTGC CCAGAAGCTG 1320

TGGATTGAGG GGCCCCCAGA GGGACAGTAC ATCCGGACTG GGACTCGGGT GAGGCTGGTA 1380

TGCTTGGCCA TCGGAGGCAA CCCAGACCCC TCCCTCATCT GGTTTAAGGA TTCACGTCCG 1440

GTGAGCGAGC CCCGGCAGCC CCAGGAGCCC CGGCGTGTGC AGCTGGGCAG TGTGGAGAAG 1500

TCCGGGAGCA CTTTCTCCCG CGAGCTGGTG TTGATCATAG GTCCGCCGGA CAACCGAGCC 1560

AAGTTCTCCT GCAAGGCGGG TCAGCTCAGT GCGTCTACGC AGCTGGTGGT GCAGTTCCCC 1620

CCAACCAACC TGACCATCCT GGCCAACTCG TCCGCGCTGC GCCCAGGCGA CGCCTTGAAC 1680

TTGACCTGCG TCAGCATCAG CAGCAACCCC CCAGTCAACT TGTCTTGGGA CAAGGAAGGA 1740

GAGAGGCTGG AAGATGTGGC TGCAAAACCC CAGAGTGCAC CGTTCAAAGG CTCCGCTGCA 1800

TCCAGGAGTG TTTTTCTCAG AGTGTCATCC CGAGACCACG GTCAACGGGT CACCTGCCGG 1860

GCCCACAGCG AGGCACTCCG TGAAACCGTG AGCTCCTTCT ACCGCTTCAA TGTGCTGTAT 1920

CCTCCAGAAT TCCTGGGGGA GCAAGTCCGG GCAGTGACCG TGGTGGAGCA GGGCCAGGTG 1980

CTGCTGCCGG TGTCGGTGTC CGCTAACCCC GCCCCCGAGG CCTTCAACTG GACCTTCCGA 2040

GGCTACCGCC TCAGCCCAGC TGGGGGTCCC CGGCACCGTA TCCTGTCTGG AGGGGCTCTG 2100

CAGCTGTGGA ATGTGACCCG AGCTGACGAT GGCTTTTATC AGCTGCACTG CCAGAACTCA 2160

GAGGGCACCG CTGAGGCGCT GTTGAAGCTG GACGTGCATT ATGCTCCCAC CATCCGTGCC 2220

CTCCGGGACC CTACTGAGGT GAATGTTGGG GGTTCTGTGG ACATAGTCTG CACCGTTGAC 2280

GCCAATCCCA TCCTCCCAGA GATGTTCAGC TGGGAGAGAC TGGGAGAAGA AGAGGAGGAT 2340

CTGAACCTGG ACGACATGGA GAAAGTTTCC AAGGGATCCA CGGGGCGTCT GCGGATTCGC 2400

CAAGCCAAGC TATCCCAGGC TGGTGCCTAC CAGTGCATCG TGGACAATGG GGTGGCTCCT 2460

GCAGCCAGAG GACTGGTTCG TCTTGTCGTC CGATTTGCTC CCCAGGTGGA TCAGCCTACT 2520

CCCCTAACAA AAGTGGCTGC CGCTGGGGAC AGCACCAGCT CAGCCACACT GCACTGCCGT 2580

GCCCGGGGTG TCCCCAACAT CGACTTCACT TGGACCAAAA ACGGGGTCCC TCTGGATCTC 2640

CAAGACCCCA GGTACACAGA GCACAGGTAC CACCAGGGTG TTGTCCACAG CAGCCTCTTG 2700

ACCATCGCTA ATGTGTCTGC GGCCCAGGAC TATGCCCTCT TCAAATGCAC GGCCACCAAT 2760

GCCCTTGGCT CTGACCACAC CAACATCCAG CTCGTCAGCA TCAGCCGCCC TGACCCTCCA 2820

CTGGGACTGA AGGTTGTCAG CATAAGCCCT CACTCGGTGG GGCTGGAGTG GAAGCCTGGC 2880

TTTGATGGGG GTCTGCCTCA GAGGTTCCAA ATCAGGTACG AGGCCCTCGA GACCCCAGGA 2940

TTCCTCCACG TGGATGTCCT ACCTACACAG GCCACTACCT TCACGCTGAC TGGGCTGAAG 3000

CCTTCTACAC GATATAGGAT CTGGCTGTTG GCCAGCAATG CCCTGGGGGA CAGTGGATTG 3060

ACGGACAAGG GGATCCAGGT CTCCGTCACT ACCCCAGGCC CCGACCAGGC TCCTGAAGAC 3120

ACAGACCACC AGCTGCCCAC AGAGCTGCCT CCAGGACCCC CAAGGCTGCC CCTGCTGCCT 3180

GTGCTCTTTG CAGTTGGTGG TCTTCTGCTG CTCTCCAATG CCTCCTGTGT TGGGGGTCTC 3240

CTCTGGCGGA GAAGACTGAG GCGCCTTGCT GAGGAGATCT CAGAGAAGAC AGAGGCAGGG 3300

TCGGAGGACA GGATCAGGAA TGAATATGAG GAGAGTCAGT GGACTGGGGA CCGGGACACG 3360

AGAAGCTCCA CGGTTAGCAC AGCAGAAGTG GACCCAAATT ACTACTCCAT GAGGGACTTC 3420

AGCCCCCAGC TTCCCCCAAC ACTGGAGGAG GTGCTGTATC ACCAAGGTGC TGAAGGCGAG 3480

GACATGGCCT TCCCCGGACA CCTGCATGAT GAAGTGGAGA GAGCCTATGG CCCGCCTGGG 3540

GCCTGGGGAC CCCTCTATGA TGAAGTACGA ATGGACCCCT ATGACCTTCG CTGGCCTGAG 3600

GTCCAATGTG AGGATCCGAG GGGAATCTAC AACCAGGTGG CAGCAGACAT GGATGCTGTG 3660

GAAGCTAGCT CTCTGCCGTT TGAGCTGAGG GGACATCTGG TGTGA 3705

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1241 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ala Leu Gly Thr Thr Leu Arg Ala Ser Leu Leu Leu Leu Gly Leu
1 5 10 15

Leu Thr Glu Gly Leu Ala Gln Leu Ala Ile Pro Ala Ser Val Pro Arg
20 25 30

Gly Phe Trp Ala Leu Pro Glu Asn Leu Thr Val Val Glu Gly Ala Ser
35 40 45

Val Glu Leu Arg Cys Gly Val Ser Thr Pro Gly Ser Ala Val Gln Trp
50 55 60

Ala Lys Asp Gly Leu Leu Leu Gly Pro Asp Pro Arg Ile Pro Gly Phe
65 70 75 80

Pro Arg Tyr Arg Leu Glu Gly Asp Pro Ala Arg Gly Glu Phe His Leu
85 90 95

Leu Ile Glu Ala Cys Asp Leu Ser Asp Asp Ala Glu Tyr Glu Cys Gln
100 105 110

Val Gly Arg Ser Glu Met Gly Pro Glu Leu Val Ser Pro Arg Val Ile
115 120 125

Leu Ser Ile Leu Val Pro Pro Lys Leu Leu Leu Leu Thr Pro Glu Ala
130 135 140

Gly Thr Met Val Thr Trp Val Ala Gly Gln Glu Tyr Val Val Asn Cys
145 150 155 160

Val Ser Gly Asp Ala Lys Pro Ala Pro Asp Ile Thr Ile Leu Leu Ser
165 170 175

Gly Gln Thr Ile Ser Asp Ile Ser Ala Asn Val Asn Glu Gly Ser Gln
180 185 190

Gln Lys Leu Phe Thr Val Glu Ala Thr Ala Arg Val Thr Pro Arg Ser
195 200 205

Ser Asp Asn Arg Gln Leu Leu Val Cys Glu Ala Ser Ser Pro Ala Leu
210 215 220

Glu Ala Pro Ile Lys Ala Ser Phe Thr Val Asn Val Leu Phe Pro Pro
225 230 235 240

Gly Pro Pro Val Ile Glu Trp Pro Gly Leu Asp Glu Gly His Val Arg
245 250 255

Ala Gly Gln Ser Leu Glu Leu Pro Cys Val Ala Arg Gly Gly Asn Pro
260 265 270

Ile Ala Thr Leu Gln Trp Leu Lys Asn Gly Gln Pro Val Ser Thr Ala
275 280 285

Trp Gly Thr Glu His Thr Gln Ala Val Ala Arg Ser Val Leu Val Met
290 295 300

Thr Val Arg Pro Glu Asp His Gly Ala Gln Leu Ser Cys Glu Ala His
305 310 315 320

Asn Ser Val Ser Ala Gly Thr Gln Glu His Gly Ile Thr Leu Gln Val
325 330 335

Thr Phe Pro Pro Ser Ala Ile Ile Ile Leu Gly Ser Ala Ser Gln Thr
340 345 350

Glu Asn Lys Asn Val Thr Leu Ser Cys Val Ser Lys Ser Ser Arg Pro
355 360 365

Arg Val Leu Leu Arg Trp Trp Leu Gly Trp Arg Gln Leu Leu Pro Met
370 375 380

Glu Glu Thr Val Met Asp Gly Leu His Gly Gly His Ile Ser Met Ser
385 390 395 400

Asn Leu Thr Phe Leu Ala Arg Arg Glu Asp Asn Gly Leu Thr Leu Thr
405 410 415

Cys Glu Ala Phe Ser Glu Ala Phe Thr Lys Glu Thr Phe Lys Lys Ser
420 425 430

Leu Ile Leu Asn Val Lys Tyr Pro Ala Gln Lys Leu Trp Ile Glu Gly
435 440 445

Pro Pro Glu Gly Gln Lys Leu Arg Ala Gly Thr Arg Val Arg Leu Val
450 455 460

Cys Leu Ala Ile Gly Gly Asn Pro Glu Pro Ser Leu Met Trp Tyr Lys
465 470 475 480

Asp Ser Arg Thr Val Thr Glu Ser Arg Leu Pro Gln Glu Ser Arg Arg
485 490 495

Val His Leu Gly Ser Val Glu Lys Ser Gly Ser Thr Phe Ser Arg Glu
500 505 510

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Leu Val Leu Val Thr Gly Pro Ser Asp Asn Gln Ala Lys Phe Thr Cys
        515                 520                 525

Lys Ala Gly Gln Leu Ser Ala Ser Thr Gln Leu Ala Val Gln Phe Pro
    530                 535                 540

Pro Thr Asn Val Thr Ile Leu Ala Asn Ala Ser Ala Leu Arg Pro Gly
545                 550                 555                 560

Asp Ala Leu Asn Leu Thr Cys Val Ser Val Ser Ser Asn Pro Pro Val
                565                 570                 575

Asn Leu Ser Trp Asp Lys Glu Gly Glu Arg Leu Glu Gly Val Ala Ala
            580                 585                 590

Pro Pro Arg Arg Ala Pro Phe Lys Gly Ser Ala Ala Ala Arg Ser Val
        595                 600                 605

Leu Leu Gln Val Ser Ser Arg Asp His Gly Gln Arg Val Thr Cys Arg
    610                 615                 620

Ala His Ser Ala Glu Leu Arg Glu Thr Val Ser Ser Phe Tyr Arg Leu
625                 630                 635                 640

Asn Val Leu Tyr Arg Pro Glu Phe Leu Gly Glu Gln Val Leu Val Val
                645                 650                 655

Thr Ala Val Glu Gln Gly Glu Ala Leu Leu Pro Val Ser Val Ser Ala
            660                 665                 670

Asn Pro Ala Pro Glu Ala Phe Asn Trp Thr Phe Arg Gly Tyr Arg Leu
        675                 680                 685

Ser Pro Ala Gly Gly Pro Arg His Arg Ile Leu Ser Ser Gly Ala Leu
    690                 695                 700

His Leu Trp Asn Val Thr Arg Ala Asp Asp Gly Leu Tyr Gln Leu His
705                 710                 715                 720

Cys Gln Asn Ser Glu Gly Thr Ala Glu Ala Arg Leu Arg Leu Asp Val
                725                 730                 735

His Tyr Ala Pro Thr Ile Arg Ala Leu Gln Asp Pro Thr Glu Val Asn
            740                 745                 750

Val Gly Gly Ser Val Asp Ile Val Cys Thr Val Asp Ala Asn Pro Ile
        755                 760                 765

Leu Pro Gly Met Phe Asn Trp Glu Arg Leu Gly Glu Asp Glu Glu Asp
    770                 775                 780

Gln Ser Leu Asp Asp Met Glu Lys Ile Ser Arg Gly Pro Thr Gly Arg
785                 790                 795                 800
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Leu Arg Ile His His Ala Lys Leu Ala Gln Ala Gly Ala Tyr Gln Cys
                805                 810                 815

Ile Val Asp Asn Gly Val Ala Pro Pro Ala Arg Arg Leu Leu Arg Leu
            820                 825                 830

Val Val Arg Phe Ala Pro Gln Val Glu His Pro Thr Pro Leu Thr Lys
        835                 840                 845

Val Ala Ala Ala Gly Asp Ser Thr Ser Ser Ala Thr Leu His Cys Arg
    850                 855                 860

Ala Arg Gly Val Pro Asn Ile Val Phe Thr Trp Thr Lys Asn Gly Val
865                 870                 875                 880

Pro Leu Asp Leu Gln Asp Pro Arg Tyr Thr Glu His Thr Tyr His Gln
                885                 890                 895

Gly Gly Val His Ser Ser Leu Leu Thr Ile Ala Asn Val Ser Ala Ala
            900                 905                 910

Gln Asp Tyr Ala Leu Phe Thr Cys Thr Ala Thr Asn Ala Leu Gly Ser
        915                 920                 925

Asp Gln Thr Asn Ile Gln Leu Val Ser Ile Ser Arg Pro Asp Pro Pro
    930                 935                 940

Ser Gly Leu Lys Val Val Ser Leu Thr Pro His Ser Val Gly Leu Glu
945                 950                 955                 960

Trp Lys Pro Gly Phe Asp Gly Gly Leu Pro Gln Arg Phe Cys Ile Arg
                965                 970                 975

Tyr Glu Ala Leu Gly Thr Pro Gly Phe His Tyr Val Asp Val Val Pro
            980                 985                 990

Pro Gln Ala Thr Thr Phe Thr Leu Thr Gly Leu Gln Pro Ser Thr Arg
        995                 1000                1005

Tyr Arg Val Trp Leu Leu Ala Ser Asn Ala Leu Gly Asp Ser Gly Leu
    1010                1015                1020

Ala Asp Lys Gly Thr Gln Leu Pro Ile Thr Thr Pro Gly Leu His Gln
1025                1030                1035                1040

Pro Ser Gly Glu Pro Glu Asp Gln Leu Pro Thr Glu Pro Pro Ser Gly
                1045                1050                1055

Pro Ser Gly Leu Pro Leu Leu Pro Val Leu Phe Ala Leu Gly Gly Leu
            1060                1065                1070

Leu Leu Leu Ser Asn Ala Ser Cys Val Gly Gly Val Leu Trp Gln Arg
        1075                1080                1085
```

Arg Leu Arg Arg Leu Ala Glu Gly Ile Ser Glu Lys Thr Glu Ala Gly
1090 1095 1100

Ser Glu Glu Asp Arg Val Arg Asn Glu Tyr Glu Glu Ser Gln Trp Thr
1105 1110 1115 1120

Gly Glu Arg Asp Thr Gln Ser Ser Thr Val Ser Thr Thr Glu Ala Glu
1125 1130 1135

Pro Tyr Tyr Arg Ser Leu Arg Asp Phe Ser Pro Gln Leu Pro Pro Thr
1140 1145 1150

Gln Glu Glu Val Ser Tyr Ser Arg Gly Phe Thr Gly Glu Asp Glu Asp
1155 1160 1165

Met Ala Phe Pro Gly His Leu Tyr Asp Glu Val Glu Arg Thr Tyr Pro
1170 1175 1180

Pro Ser Gly Ala Trp Gly Pro Leu Tyr Asp Glu Val Gln Met Gly Pro
1185 1190 1195 1200

Trp Asp Leu His Trp Pro Glu Asp Thr Tyr Gln Asp Pro Arg Gly Ile
1205 1210 1215

Tyr Asp Gln Val Ala Gly Asp Leu Asp Thr Leu Glu Pro Asp Ser Leu
1220 1225 1230

Pro Phe Glu Leu Arg Gly His Leu Val
1235 1240

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Gly Pro Ser Gly Leu Pro Leu Leu Pro Val Leu Phe Ala Leu Gly Gly
1 5 10 15

Leu Leu Leu Leu Ser Asn Ala Ser Cys Val Gly Gly Val Leu Trp Gln
20 25 30

Arg Arg Leu Arg Arg Leu
35

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Leu Pro Thr Glu Pro Pro Ser Gly Ile Ser Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTGCCCACAG AGCCACCTTC AGGCATCTCA GAG 33

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GACAAAGCCA GACAGACGCA G 21

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AGCTTCCGC 9

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Glu Asp Gln Leu Pro Thr Glu Pro Pro Ser Gly Ile Ser Glu Lys Thr
1 5 10 15

Glu Ala Gly Ser Glu
20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Leu His Gln Pro Ser Gly Glu Pro Glu Asp Gln Leu Pro Thr Glu Pro
1 5 10 15

Pro Ser Gly Ile Ser Glu Lys Thr Glu Ala Gly Ser Glu Glu Asp Arg
20 25 30

Val Arg Asn Glu
35

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Leu Pro Ile Thr Thr Pro Gly Leu His Gln Pro Ser Gly Glu Pro Glu
1 5 10 15

Asp Gln Leu Pro Thr Glu Pro Pro Ser Gly Ile Ser Glu Lys Thr Glu
20 25 30

Ala Gly Ser Glu Glu Asp Arg Val Arg Asn Glu Tyr Glu Glu Ser Gln
35 40 45

Trp

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Ala Asp Lys Gly Thr Gln Leu Pro Ile Thr Thr Pro Gly Leu His Gln
1 5 10 15

Pro Ser Gly Glu Pro Glu Asp Gln Leu Pro Thr Glu Pro Pro Ser Gly
20 25 30

Ile Ser Glu Lys Thr Glu Ala Gly Ser Glu Glu Asp Arg Val Arg Asn
35 40 45

Glu Tyr Glu Glu Ser Gln Trp Thr Gly Glu Arg Asp Thr
50 55 60

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Ala Ser Asn Ala Leu Gly Asp Ser Gly Leu Ala Asp Lys Gly Thr Gln
1 5 10 15

Leu Pro Ile Thr Thr Pro Gly Leu His Gln Pro Ser Gly Glu Pro Glu
20 25 30

Asp Gln Leu Pro Thr Glu Pro Pro Ser Gly Ile Ser Glu Lys Thr Glu
35 40 45

Ala Gly Ser Glu Glu Asp Arg Val Arg Asn Glu Tyr Glu Glu Ser Gln
50 55 60

Trp Thr Gly Glu Arg Asp Thr Gln Ser Ser Thr Val Ser Thr
65 70 75

We claim:

1. An isolated nucleic acid sequence encoding nephrin or soluble nephrin-like molecules shared by pancreas and kidney glomerulus, c h a r a c t e r i z e d in, that it comprises the nucleic acid sequence SEQ ID NO:1: or nucleic acid sequences with substantial similarity encoding nephrin-like molecules or derivatives thereof, having the properties and functions characteristic of nephrin-like molecules, said nephrin-like molecules having an amino acid sequence substantially homologous with SEQ ID NO:2: but lacking the transmembraneous domain SEQ ID NO:3: of nephrin.

2. The isolated nucleic acid sequence according to claim 1, c h a r a c t e r i z e d in, that it comprises the nucleic acid sequence SEQ ID NO:1: or nucleic acid sequences with substantial similarity encoding nephrin-like molecules or derivatives thereof, having the properties and functions characteristical of nephrin-like molecules, said nephrin-like molecules having an amino acid sequence substantially homologous with SEQ ID NO:2: but lacking the transmembraneous domain SEQ ID NO:3: and having at least one contagious amino acid sequence (SEQ ID NO:4:).

3. The isolated nucleic acid sequence according to claim 1, c h a r a c t e r i z e d in, that it comprises the human nephrin encoding nucleic acid sequence SEQ ID NO:1:.

4. Soluble nephrin-like molecules shared by pancreas and kidney glomerulus, c h a r - a c t e r i z e d in, that they comprise polypeptides or derivatives thereof, having the properties and functions characteristical of nephrin-like molecules and being substantially homologous with the amino acid sequence SEQ ID NO:2: but lacking the transmembraneous domain SEQ ID NO:3: of nephrin.

5. The soluble nephrin-like molecules according to claim 4, c h a r a c t e r i z e d in, that they comprise at least one contagious amino acid sequence (SEQ ID NO:4:).

6. A binding substance, c h a r a c t e r i z e d in, that it is capable of specifically recognizing and binding to the soluble nephrin-like molecules according to claims 4-5 or nucleic acid sequences encoding such nephrin-like molecules.

7. The use of the isolated nucleic acid sequences according to claims 1-3 for manufacturing means for diagnostic evaluation, prophylactic and therapeutic treatment of

diabetic and other nephropathies and of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases.

8. The use of the soluble nephrin-like molecules according to claim 3-4 or parts thereof for screening or for manufacturing of test kits for screening the presence or absence of autoantibodies against said soluble nephrin-like molecules in order to assess the susceptibility of a person to diabetic and other nephropathies and of diabetes mellitus and other inflammatory and neoplastic pancreatic disease.

9. The use of the soluble nephrin-like molecules according to claim 3-4 or parts thereof in the production of means for drug and therapy development of diabetic and other nephropathies and of diabetes mellitus and other inflammatory and neoplastic pancreatic disease.

10. The use of the binding substances according to claim 6 for diagnostic evaluation and for manufacturing of means for diagnostic evaluation, prophylactic and therapeutic treatment of diabetic and other nephropathies and of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases.

Abstract

The present invention is related to the of soluble nephrin-like molecules expressed by pancreas and kidney and nucleic acid sequences encoding said nephrin-like molecules, including a human nephrin encoding cDNA, as well as binding substances, capable of specifically recognizing and binding said nephrin-like molecules. The molecules are useful for manufacturing means for diagnostic determination, screening of susceptibility, prophylactic and therapeutic treatment of diabetic and other nephropathies and of diabetes mellitus and other inflammatory and neoplastic pancreatic diseases.

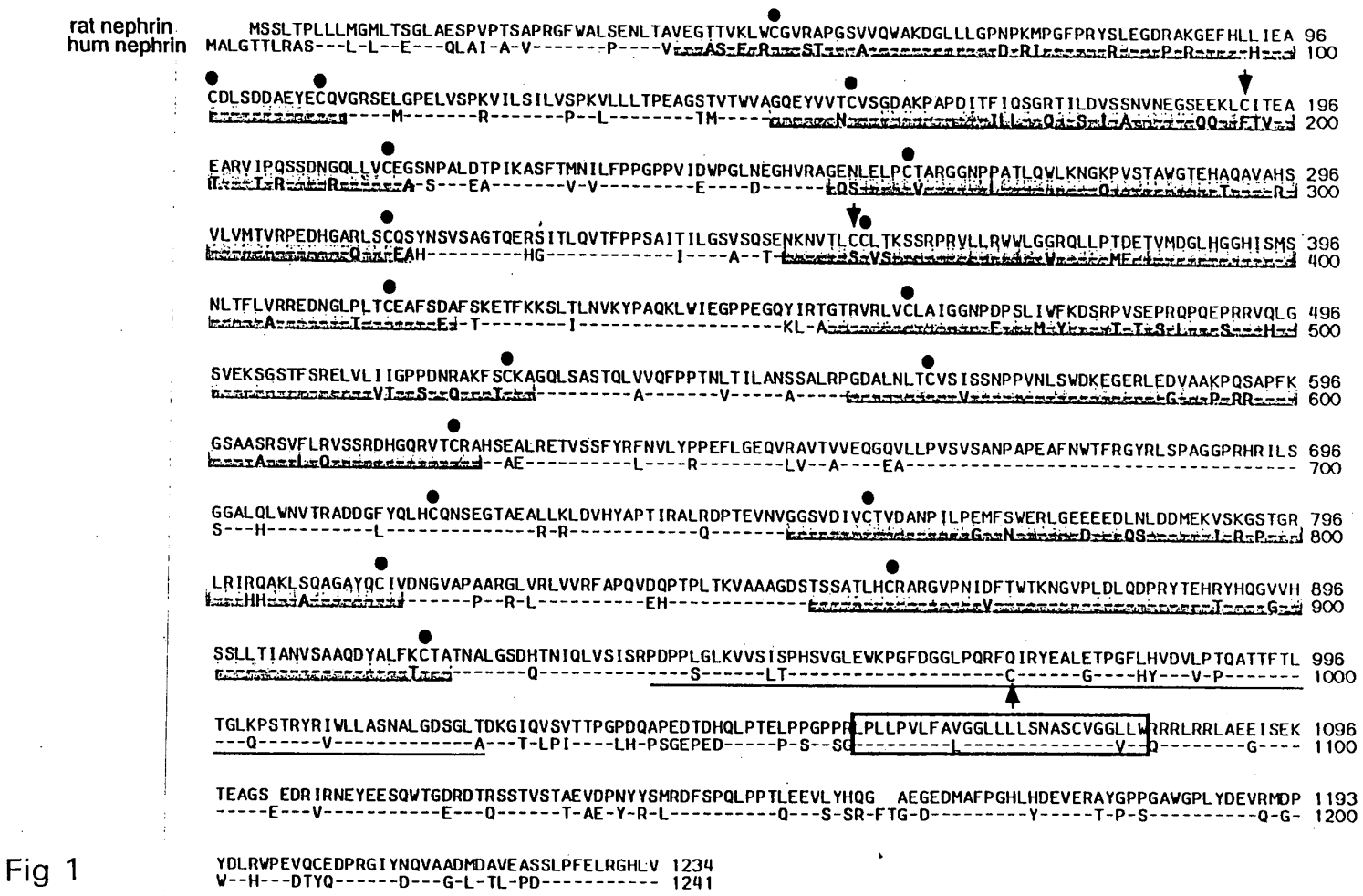

Fig 1

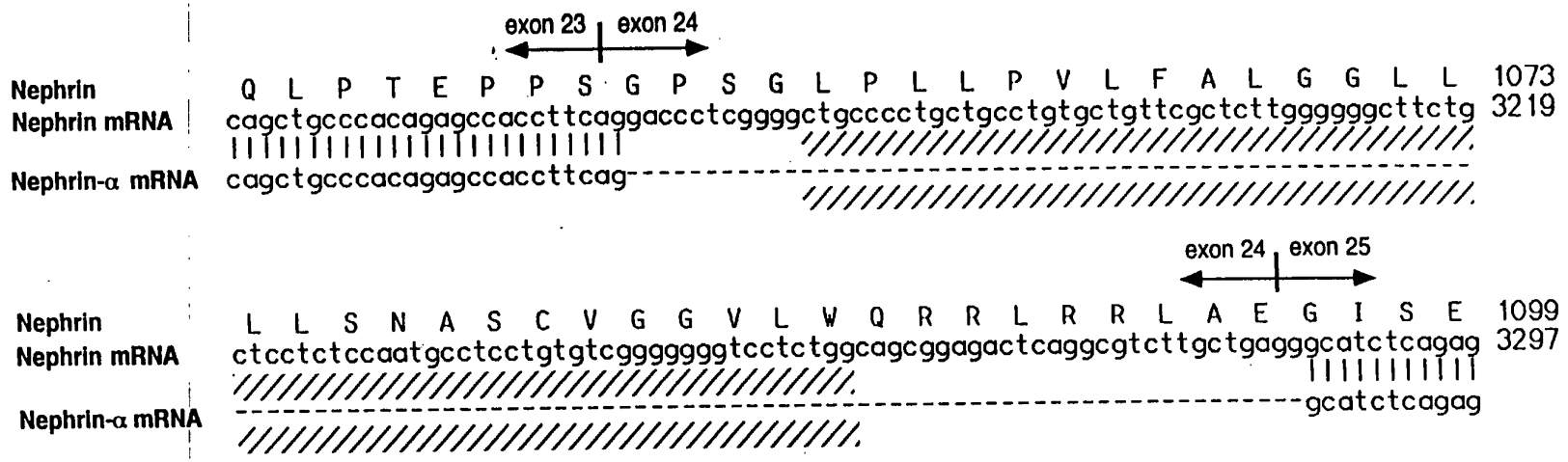
exon 23
exon 24
Nephrin
Q L P T E P P S G P S G L P L L P V L F A L G G L L 1073
Nephrin mRNA
cagctgcccacagagccaccttcaggaccctcggggctgcccctgctgcctgtgctgttcgctcttgggggcttctg 3219
Nephrin-α mRNA
cagctgcccacagagccaccttcag
exon 24
exon 25
Nephrin
L L S N A S C V G G V L W Q R R L R R L A E G I S E 1099
Nephrin mRNA
ctcctctccaatgcctcctgtgtcgggggggtcctctggcagcggagactcaggcgtcttgctgagggcatctcagag 3297
Nephrin-α mRNA
gcatctcagag

Fig 2

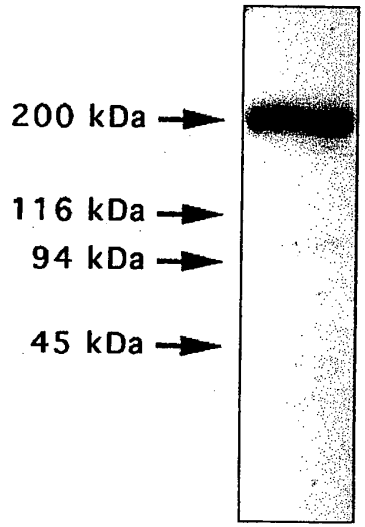

Fig 3